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Kinetics of the Natural, Humoral Immune Response to *Salmonella enterica* Serovar Typhi in Kathmandu, Nepal[▽]

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Typhoid fever is a major public health problem in developing countries, conservatively estimated to occur in 17 million cases and be responsible for 200,000 deaths annually. We investigated the acquisition of natural immunity to *Salmonella enterica* serovar Typhi in a region where typhoid is endemic by testing sera from an age-stratified sample of 210 healthy participants in Kathmandu, Nepal, for bactericidal activity toward *S.* Typhi and for anti-Vi capsular polysaccharide antibodies. Bactericidal titers in children were significantly lower than those in newborns and adults ($P < 0.0001$). Anti-*S.* Typhi bactericidal geometric mean titers were age dependent, increasing 10-fold during childhood. Anti-Vi polysaccharide antibody geometric mean concentrations were also lower in children than in adults. Data presented here indicate the possibility of a relationship between low levels of bactericidal activity toward *S.* Typhi in serum and susceptibility to disease, as observed for other polysaccharide-encapsulated bacteria. Bactericidal antibody may be a marker of protective immunity against *S.* Typhi.

Salmonella enterica subspecies *enterica* serotype Typhi is responsible for 17 million new cases of typhoid fever and 200,000 deaths annually (5). Since typhoid fever is a food- and waterborne disease transmitted by the fecal-oral route, it is endemic in regions where the quality of the drinking water supply is poor and sewage disposal facilities are inadequate, with annual incidence rates above 100 per 100,000 person years (2). Southern Asia carries a heavy burden of disease, with *S.* Typhi causing almost 40% of culture-positive bloodstream infections in Nepal (26). Although typhoid was thought to be a disease of school children and young adults, there is increasing evidence of a substantial disease burden in younger children in countries where typhoid is endemic (3, 24, 27).

Three vaccines for the prevention of typhoid fever exist. The whole-cell inactivated vaccine is unpopular due to high rates of adverse reactions (19). A live attenuated oral vaccine (*Ty21*a) and a parenteral plain polysaccharide vaccine have comparable efficacies ranging between 50 and 70% and are suitable for community vaccination programs in areas of hyperendemicity (8, 9). A polysaccharide-protein conjugate vaccine has shown promising results in clinical trials and potentially could be used for children under 2 years of age, for whom the former two vaccines are either ineffective or unlicensed (17, 21). Similarly, a novel, drinkable, single-dose attenuated vaccine has demonstrated promising immunogenicity and tolerability in phase II trials in Vietnam (31). Despite the availability of two moderately efficacious vaccines and the possibility of another two, no country has implemented a systematic typhoid vaccination strategy. This is due partly to the limited data on disease burden and age-specific immunity, which are critical in determining the timing of and need for vaccination (6). Today, typhoid immunization is limited to travelers visiting regions of endemicity or laboratory workers potentially exposed to the pathogen.

We investigated anti-*S.* Typhi bactericidal antibody titers and the concentrations of antibody against the *S.* Typhi Vi polysaccharide (ViPS) in a cross-sectional study of a population in Kathmandu, Nepal, in order to relate antibody levels to both age and reported disease rates.

MATERIALS AND METHODS

Participants and samples. Venous blood samples (10 ml) were obtained with informed consent from consecutive individuals attending the outpatient department of Patan Hospital in Lalitpur, Kathmandu, Nepal, during June and July 2006. Cord blood samples were obtained from consecutive deliveries in the maternity ward at the hospital when consent was provided. Volunteers who had a history of culture-confirmed enteric fever, had received a typhoid vaccine, or were febrile or receiving antibiotics or immunomodulators were excluded. Sera were separated from clotted whole-blood samples by centrifugation prior to being frozen and transported to the United Kingdom for analysis.

A control group consisting of 180 anonymous samples from individuals in the

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United Kingdom, including newborns and participants up to 55 years of age, was obtained. The sera had been collected from healthy individuals participating in vaccine trials in Oxfordshire. The study protocol was approved by the Oxford Tropical Research Ethics Committee (OxTREC no. 017-05), the Oxfordshire Research Ethics Committee, and the Nepal Health Research Council.

SBA assay. Bactericidal activity was determined by means of a novel serum bactericidal antibody (SBA) assay. In brief, sera decomplemented at 56°C for 30 min and subjected to a series of twofold dilutions, from 1:4 up to 1:1,024, were incubated in Hanks balanced salt solution (Sigma, United Kingdom) with 250 CFU of *S.* Typhi (NCTC *Ty2* strain) and a 1:8 dilution of freshly thawed baby rabbit complement (Pel-Freez Biologicals) at 37°C for 45 min before overnight incubation on Luria-Bertani agar plates. The inverse of the highest dilution factor at which ≥50% of bacteria, relative to the bacteria in a control well (T45), were killed was considered to be the bactericidal titer for that sample. Results for three assay controls for each sample, including a viable-cell count control (containing no serum or complement but Hanks balanced salt solution and *S.* Typhi, to serve as a bacterial colony count control), a complement-independent control (containing no complement but serum and *S.* Typhi, to detect intrinsic serum bactericidal activity), and a complement control (containing no serum but complement and *S.* Typhi, to detect bacterial complement sensitivity), confirmed the validity of the bactericidal activity. All samples were analyzed in duplicate, and the average results were recorded.

Reference ELISA for IgG concentration. In the absence of a standard reference serum for anti-ViPS antibody, a reference enzyme-linked immunosorbent assay (ELISA) was designed to establish the absolute antibody titer for a control serum obtained from a volunteer who had received the ViPS vaccine previously. This technique is based on the principle that equivalent absorbance levels for samples in two ELISAs performed in parallel under identical assay conditions represent equivalent amounts of antibodies when results are corrected for concentration (22). In brief, European reference material DA470, containing 9.68 g/liter of total immunoglobulin G (IgG), was subjected to serial twofold dilutions starting at 1:100,000, and diluted samples were run simultaneously with serial twofold dilutions of the test serum starting at 1:25. The rest of the protocol for the total-IgG ELISA described below was followed. Optical densities (ODs) obtained for both ELISAs were plotted concurrently, and the absolute antibody titer for the test serum was estimated by correcting for the dilution factor.

ViPS ELISA. *S.* Typhi ViPS-specific IgG concentrations were determined using a novel ELISA. In brief, Maxisorp plates (Nunc) were coated with 100 μl of ViPS (Sanofi-Pasteur, France) at a concentration of 5 μg/ml in a diluent solution containing sodium chloride, potassium chloride, magnesium sulfate, and calcium chloride at a physiological pH. After overnight incubation at 4°C and blocking with 2% skim milk powder (Sigma, United Kingdom), duplicates of a 1:25 dilution of serum were incubated for 2 h. Additionally, three dilutions (1:25, 1:200, and 1:800) of an internal quality control serum were run on each plate to confirm that the ODs of these samples were in the high, medium, and low ranges. A reference serum, the antibody titer of which was determined by the reference ELISA method described above, was used for the estimation of absolute IgG titers in the test sera. The plate was developed with anti-human IgG γ chain conjugated with peroxidase (diluted in serum/conjugate buffer; Sigma, United Kingdom) for an hour, followed by the chromogenic substrate tetramethylbenzidine dihydrochloride monohydrate (Sigma, United Kingdom), and the reaction was stopped after 15 min with 2 M sulfuric acid. The OD of each well at 450 nm was then read.

ViPS IgG subclass ELISA. To determine the anti-ViPS IgG subclass profile, the protocol for the ViPS ELISA was modified to include conjugated IgG subclass secondary antibody per the instructions of the manufacturer (Sigma, United Kingdom). Profiles for 28 Nepal samples, including 4 samples from each of the seven age groups, all with total-IgG ELISA results showing high antibody concentrations, were analyzed and compared to the IgG subclass profile for a post-ViPS vaccination serum sample.

Statistical analyses. Analyses of results from this cross-sectional observational study were primarily descriptive. A sample size of 457 was calculated to detect a 20% difference in antibody titer within the study population, with a 95% confidence level. SBA titers and ELISA IgG concentrations are summarized as geometric means with corresponding 95% confidence intervals (95% CI). One-way analysis of variance was employed to detect significant differences in bactericidal geometric mean titer (GMT) among the age groups within each cohort. Pearson correlation was performed to detect correlation between the bactericidal GMT and the anti-ViPS IgG level, expressed as the geometric mean concentration (GMC).

RESULTS

Participants and samples. A total of 458 samples were obtained from study participants in Nepal. Two hundred ten samples from Nepal, including 30 random samples from each of the seven age groups in the Nepal cohort, and 180 samples from the United Kingdom, including 30 samples from each of the six age groups in the United Kingdom cohort, were initially tested and analyzed.

SBA assay. Geometric mean bactericidal antibody titers in cord blood samples from the Nepal cohort were high but had fallen by 6 months after birth, reaching adult levels in the second decade of life (Fig. 1). The GMTs differed significantly among the age groups ($P < 0.001$) according to one-way analysis of variance. Proportions of participants with low (<4, 4, 8, and 16), medium (32, 64, and 128), and high (256, 512, and >1,024) titers in each age group are shown in Fig. 2, which demonstrates the proportions of subjects with high or medium titers increasing with age. In the United Kingdom cohort, the geometric mean bactericidal antibody titers in sera from all age groups were low and the GMTs did not differ significantly among the age groups (data not shown).

Reference ELISA. At an OD of 0.6, with correction for the dilution factors of 100 and 800,000 for the test serum and reference serum, respectively, the absolute antibody concentration in the test serum was determined to be 1.21 μg/ml (Fig. 3). This concentration, arbitrarily selected at a particular OD factor to coincide with the midpoint of the linear phase of the OD curve, and the concentration in the reference serum served as the reference points for estimating absolute concentrations in all other sera in the study.

ViPS ELISA. The GMCs of the anti-ViPS antibody are shown in Fig. 4. A significant correlation between the bactericidal GMT and the geometric mean anti-ViPS antibody concentration was observed for the United Kingdom cohort ($r = 0.92$) but not for the Nepal cohort ($r = 0.07$).

ViPS IgG subclass ELISA. Similar to the IgG subclass profile for the postvaccination serum, the profiles for sera from all age groups in the Nepal cohort showed IgG2 predominating in the anti-ViPS antibody response (Fig. 5).

DISCUSSION

This study provides the first description of the acquisition of natural humoral immunity to *S.* Typhi in an age-stratified population from a region where typhoid is endemic. Although the mechanisms or correlates of protection against typhoid fever are unknown, given the protection offered by the antibody-inducing ViPS vaccine, circulatory antibody may have a role in mediating protection against typhoid fever (1). Both secretory IgA and cell-mediated immunity induced by the live attenuated vaccine are thought to be protective (30). Therefore, a combination of different mechanisms may contribute to immunity against *S.* Typhi following natural exposure or vaccination. The relative importance of each mechanism remains unknown.

We hypothesized that natural immunity to typhoid fever is age dependent and related to subclinical exposure to *S.* Typhi in populations living in countries where typhoid is endemic, and our results confirm that natural immunity is acquired with age. The high titers of bactericidal antibody at birth reflect the

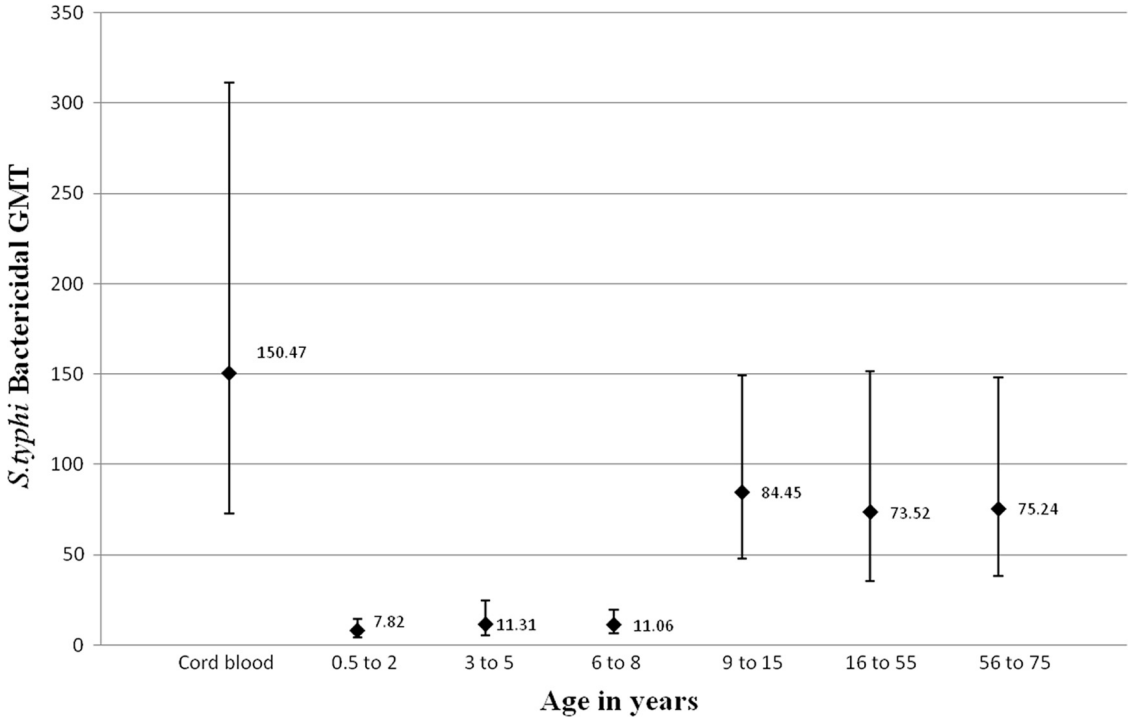

FIG. 1. Age-specific *S*. Typhi bactericidal GMTs (95% CIs) in the Nepal cohort.

transplacental transfer of maternal antibody. The low titers in later infancy presumably reflect the loss of maternal antibody and the lack of exposure of the breast-feeding infant to contaminated water. The SBA GMT remains low during the first decade of life, rising to adult levels during adolescence. The mechanism by which the cord blood bactericidal antibody GMT is almost twofold higher than the adult titers is not known. This finding may simply reflect a selection bias if mothers who deliver at the hospital are from a population that differs from those we targeted for the other age groups. Alternatively, there may be some systematic differences in the bactericidal activity of maternal sera. However, to comment on

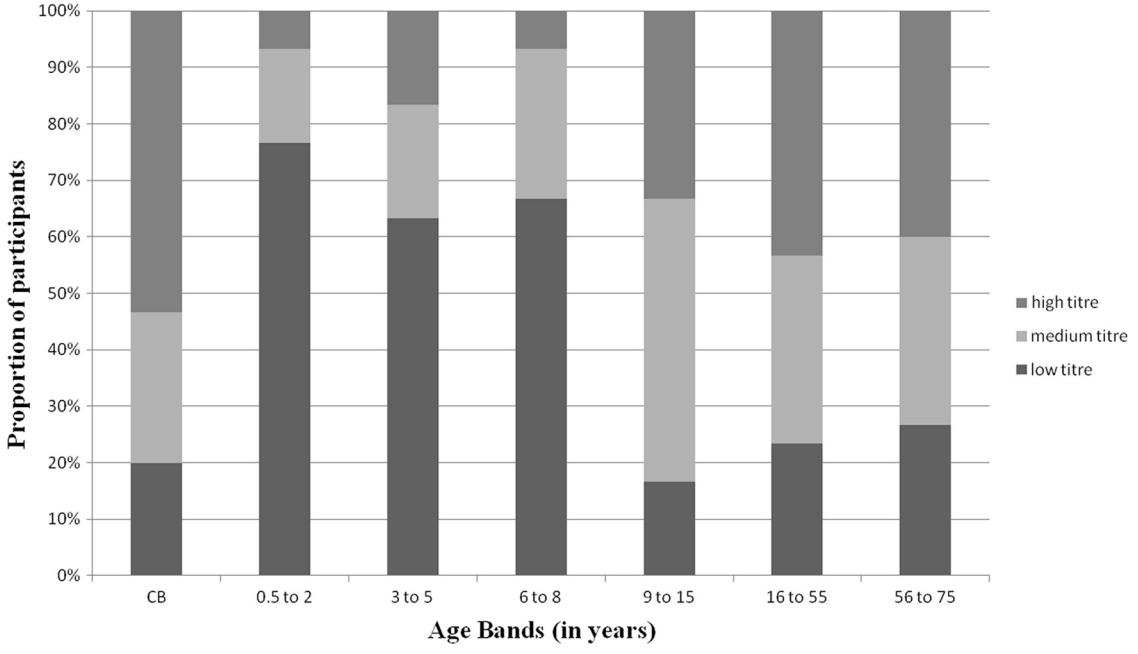

FIG. 2. Age-specific proportions of high (≥256), medium (32 to 128), and low (≤16) *S.* Typhi bactericidal titers in the Nepal cohort. CB, cord blood sera.

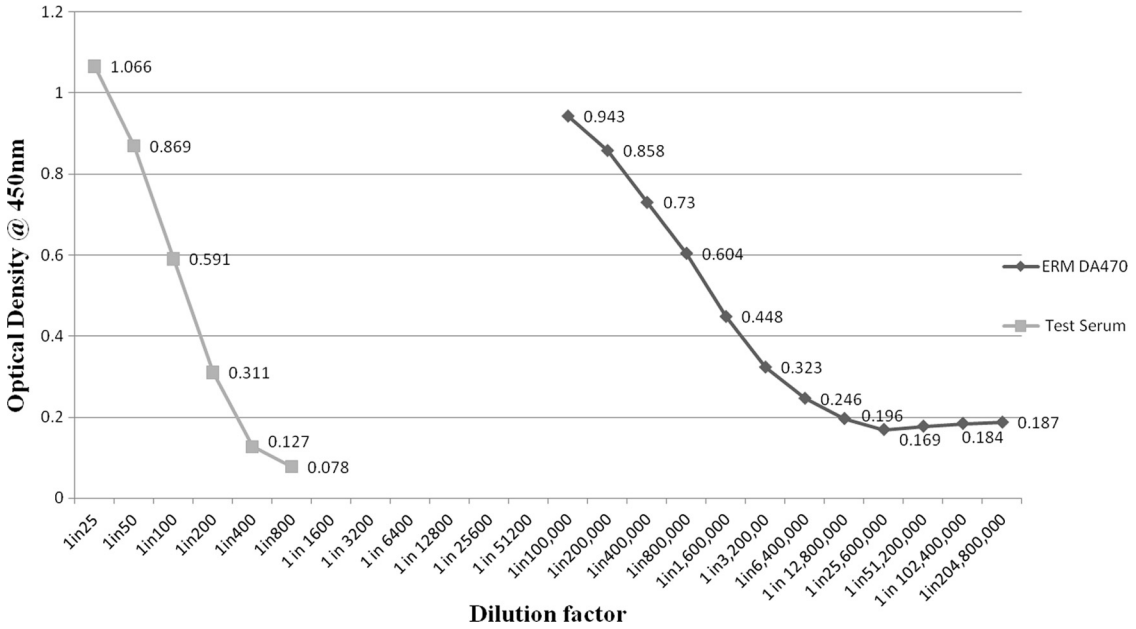

FIG. 3. Results from a reference ELISA comparing the ODs obtained with *S.* Typhi anti-ViPS IgG (test serum) and European reference material (ERM) DA470.

whether this additional bactericidal activity actually translates into clinical protection is beyond the scope of this study.

Using the data from the prospective follow-up study by Sinha et al. (27), we are able to show an inverse relationship between the age-related rates of typhoid fever and the SBA GMT, indicating the possibility that bactericidal activity in serum may correlate with protection against typhoid fever. However, we do not have direct evidence that individuals who develop typhoid have lower bactericidal titers than those who do not, and our observation does not therefore prove susceptibility. Furthermore, we do not have rates of typhoid fever for the urban population of Kathmandu and have based this relationship on data collected from a region in neighboring India.

S. Typhi is an enteric pathogen that is transmitted through contaminated food and water, and greater exposure during early school age from the consumption of contaminated food

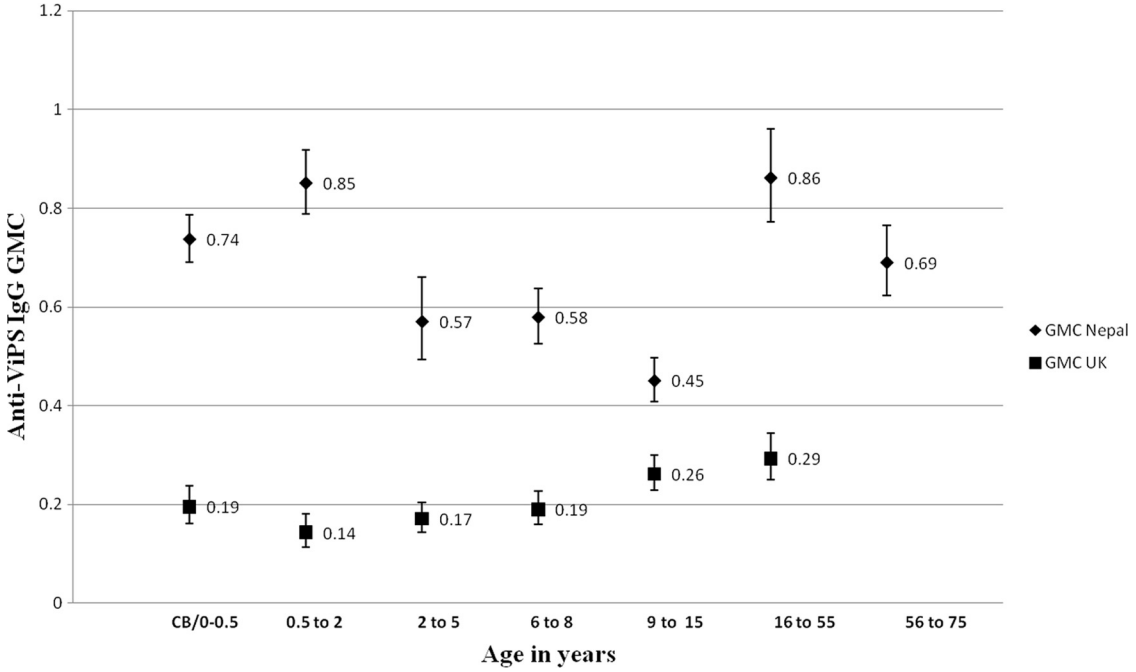

FIG. 4. Anti-ViPS antibody GMCs in the Nepal and United Kingdom cohorts (95% CI). CB, cord blood sera.

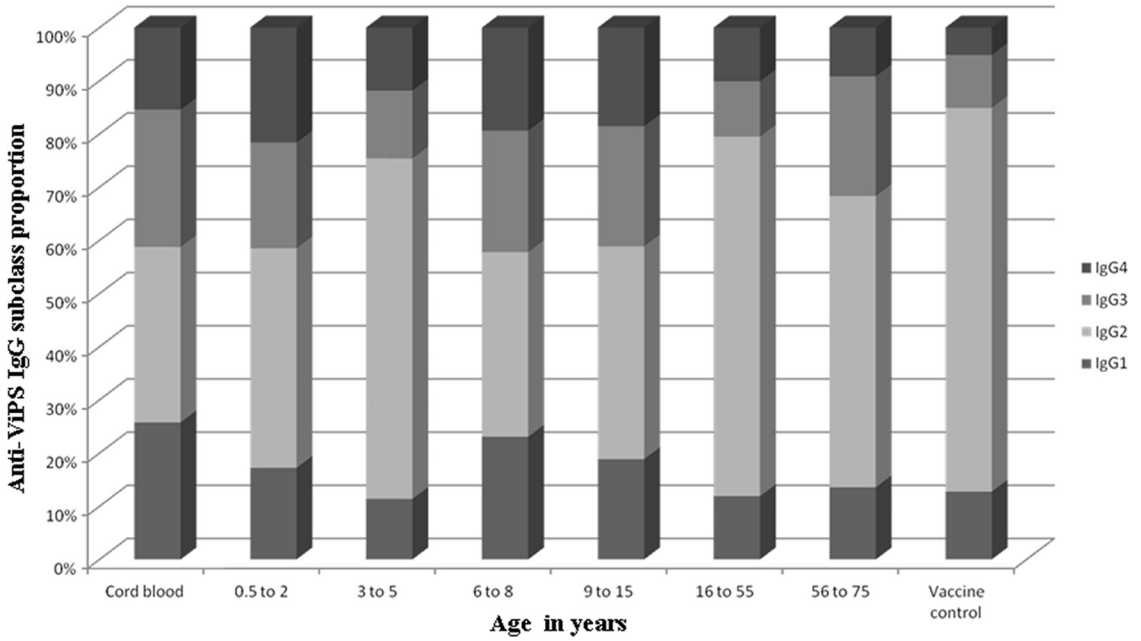

FIG. 5. IgG subclass of anti-ViPS antibody within each age group of the Nepal cohort (ages are expressed in years) and IgG subclass profile of a post-Vi vaccination control serum.

and water outside of the home may explain the increase in bactericidal activity with age. While it is expected that subclinical exposure should begin to occur in early school age, the observed rise in bactericidal titer beginning around 9 years of age may suggest, in addition to subclinical exposure to *S.* Typhi as the probable reason for natural immunity, that a certain duration of such exposure is needed prior to the attainment of critical bactericidal activity or that this attainment occurs through the maturation of immune responses around age 9, as has been suggested for *Neisseria meningitidis* serogroup C SBA (28). The presence of relatively high bactericidal titers in older children and adults, again possibly as a consequence of subclinical exposure, may explain the low incidence of typhoid fever in these age groups in regions where the disease is endemic.

An inverse relationship between the increase in bactericidal antibodies against *Haemophilus influenzae* in children between 2 and 3 years of age and a decrease in the incidence of invasive *H. influenzae* type b disease was first reported by Fothergill and Wright in 1933 (7). Goldschneider et al., in a seminal paper published in 1969, demonstrated that susceptibility to meningococcal disease is inversely related to the presence of SBA (10). This conclusion was determined on the basis of results from a study of military recruits, using an SBA assay, which found that individuals with naturally acquired titers of ≥4 were protected from meningococcal serogroup C disease. In the absence of vaccination, SBAs were generated naturally following periods of meningococcal carriage and were also obtained through transplacental acquisition. The nadir in the protective bactericidal titer occurred in children at 12 months of age, the point at which the highest incidence of disease was found, from which time it consistently increased to achieve adult titers by mid-teen age (11).

A possible explanation for the increase in *H. influenzae* and *N. meningitidis* bactericidal titers after the first year of life, in contrast to the increase in *S.* Typhi titers in children at 9 years of age, may be that *H. influenzae* and *N. meningitidis* are airborne pathogens and that nasopharyngeal colonization and carriage occur earlier in life, with the resulting natural immunity, than exposure to *S.* Typhi, which may be delayed until children reach early school age due to the reasons mentioned above.

For *N. meningitidis* serogroup C, the correlate of protection in terms of the bactericidal titer has been estimated to be ≥4 by using human complement, but the titer beyond which protection is afforded against *S.* Typhi is unknown. Hence, in the absence of an established threshold for protection, the determination of the proportion of the population that possesses protective antibody titers as a result of subclinical exposure is not possible. However, the median bactericidal titers were 1:128 for those over 9 years of age, at which point the disease incidence is known to fall, and 1:12 for children under 8 years, the age group that has the highest disease incidence, indicating the possibility that the protective antibody titer may be more than 1:12 or, practically, considering the double dilution method used in SBA assays, the titer nearest to 1:12, i.e., 1:16.

For *H. influenzae*, the anti-polyribosylribitol phosphate antibody concentrations correlating with short-term and long-term protection are reported to be 0.15 and 1.0 μg/ml, respectively (13). Although similar putative protective antibody concentrations for *S.* Typhi are unavailable, Keitel et al. and Klugman et al. suggested, on the basis of findings from ViPS vaccine trials in which vaccine protective efficacy and anti-ViPS antibody concentrations in vaccinees were computed, that an absolute antibody titer between 0.6 to 1.5 μg/ml may be the protective level (14, 15). Furthermore, they suggested that given this cutoff, 64% of vaccinees and 40% of controls at the trial mean age of 9 years in the study population in Cape Town,

South Africa, had protective concentrations, those in the latter group due presumably to the natural acquisition of antibodies (15). Interestingly, in our study, 83.33% of adults (15 to 75 years) had an anti-ViPS antibody level of >0.6 μg/ml. IgG2 predominated in the anti-ViPS antibody responses in all age groups in the Nepal cohort. This finding fits in with the fact that IgG2 predominates in antibody responses to polysaccharide antigens.

We did not find a correlation between the bactericidal titer and the anti-ViPS concentration in the Nepal population. Antibodies against other immunodominant antigens (18, 23), e.g., the somatic O antigen, iron-regulated outer membrane proteins (4), and porins (25), may account for the predominant bactericidal activity in SBA assays. Alternately, the pattern of ViPS expression, which is strictly regulated by multiple operons, is found in response to environmental stressors, and does not occur in the bacterial intracellular niche and in culture in artificial medium, may explain the discrepancy. This explanation is strengthened by observations from other studies that showed only 20% of patients with acute typhoid fever developing anti-Vi antibodies (16). Finally, besides the absolute antibody concentrations, other qualitative factors like antibody avidity and Ig subclass that determine antibody functionality may explain the noncorrelation of antibody concentration with bactericidal titers. Previous studies of the seroprevalence of typhoid antibodies have utilized mostly the somatic O antigen and the flagellar H antigen in the Widal test, but not bactericidal activity (12, 20, 29, 32).

The observations in this study that the bactericidal activity of serum increases with age and that there may be an inverse relationship with disease incidence lend support to the development of vaccines that generate bactericidal antibody in the susceptible population of individuals in early childhood. The assessment of bactericidal activity in serum following vaccination with the currently available vaccines has not been performed. If the bactericidal activity in serum does indeed correlate with protective immunity, it remains unclear how antibody-mediated bactericidal killing is able to prevent a predominantly intracellular infection. Further exploration of mechanisms of immunity and the pathophysiology of infection, perhaps in the human challenge model, could confirm this relationship.

This seroepidemiological study provides evidence of age-dependent acquisition of natural immunity to typhoid fever that appears to correlate with disease susceptibility. If supported by the results of further studies, this finding of a correlate of immunity would drive more rapid development of new vaccines and implementation for those who are most susceptible to disease and lead to a reduction in the enormous burden of typhoid fever cases and deaths in regions where the disease is endemic.

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The views expressed in this publication are those of the authors and not necessarily those of the Department of Health.

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